FEBS Letters 582 (2008) 2387–2392

Rerouting of fibroblast growth factor 2 to the classical secretory pathway results in post-translational modifications that block binding to heparan sulfate proteoglycans

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Received 13 March 2008; revised 2 May 2008; accepted 26 May 2008

Available online 5 June 2008

Edited by Lukas Huber

Abstract FGF-2 is a proangiogenic growth factor secreted by unconventional means. It is unknown why FGF-2 takes an ER/Golgi-independent secretory route. We find that secretion of FGF-2 via the ER/Golgi system causes post-translational modifications that prevent binding to heparan sulfate proteoglycans (HSPGs), an interaction that is critically important for both FGF-2 storage and signal transduction. This loss of function is due to artificial *O*-glycosylation mainly resulting in the addition of glycosaminoglycan chains of the chrondroitin sulfate type. Our findings suggest that the unconventional mechanism of FGF-2 export is an ancient pathway of protein secretion that, in the course of evolution, has been kept due to the inability of the classical secretory pathway to export FGF-2 in a functional form.

Keywords: Unconventional protein secretion; Non-classical export; Protein targeting; Fibroblast growth factor 2; Glycosylation; Glycosaminoglycans; Heparan sulfate proteoglycans; Chondroitin sulfate

1. Introduction

Unconventional secretory proteins are defined by the ability to exit cells by mechanisms that do not depend on the endoplasmic reticulum (ER) and the Golgi apparatus [1–4]. Export of FGF-2 has been shown to be mediated by direct translocation across plasma membranes in an ATP-independent manner [5]. From these findings, it has been concluded that FGF-2 membrane translocation is a diffusion-controlled process that might be facilitated by a classical transporter or by alternative means [6]. Directional net transport of FGF-2 into the extracellular space depends on FGF-2 recruitment by phosphatidylinositol-4,5-bisphosphate at the inner leaflet of plasma membranes and a molecular trapping mechanism based on cell surface HSPGs [7,6,8]. These data are consistent with observations indicating that FGF-2 remains folded during transport across plasma membranes [9].

Here we addressed the question of why FGF-2 is not secreted by the ER/Golgi-dependent secretory pathway. Using GFP fusion proteins, we systematically compared the extracellular populations of the normal form of FGF-2, a signal-peptide-containing form of FGF-2 (SP-FGF-2) as well as FGF-4, a relative of FGF-2 that naturally contains a signal peptide and is secreted via the ER/Golgi-dependent secretory pathway. We found that the secreted population of the normal form of FGF-2 is quantitatively retained on cell surfaces where it is bound to HSPGs. By contrast, both SP-FGF-2 and FGF-4 were released in substantial amounts into the medium of cells. As opposed to FGF-2, the secreted forms of both SP-FGF-2 and FGF-4 were glycosylated. While the vast majority of secreted SP-FGF-2 contained glycosaminoglycan chains of the chondroitin sulfate type, FGF-4 was characterized by N-linked sugars. The consequences of these modifications were quite different as we found that SP-FGF-2 neither binds to heparin nor to HSPGs. By contrast, *N*-glycosylated FGF-4 efficiently bound to both heparin and HSPGs. The combined data presented in this study suggest that FGF-2 secreted by the ER/Golgi-dependent secretory pathway can neither be stored and protected by HSPGs within the extracellular matrix and on cell surfaces nor can it be functional in terms of HSPG-dependent signal transmission as part of ternary complexes involving FGF-2, HSPGs and FGF receptors.

2. Results

2.1. Addition of a signal peptide to FGF-2 results in secretion of post-translationally modified forms that are not retained by cell surface HSPGs

Using a well-characterized model system based on CHO cells and GFP fusion proteins, we have characterized the extracellular populations of FGF-2, SP-FGF-2 and FGF-4 with regard to export efficiency, post-translational modifications as well as binding to heparin and cell surface HSPGs. We found FGF-2 on cell surfaces, however, when FGF-2 was expressed in CHO mutant cells that do not make HSPGs (CHO-745; [10]), cell surface staining was almost completely abolished (Fig. 1, panel A, note log scale of *y-axis*). These findings are consistent with our earlier observations demonstrating that CHO-745 cells cannot secrete FGF-2 [7]. The majority of FGF-2 could be eluted from the surfaces of CHO wild-type cells by heparin (Fig. 2, panel B). Similar results were obtained for FGF-4, however, in case of CHO-745 cells, the cell surface signal was only reduced to about 20% as compared to CHO wild-type cells (Fig. 1, panel A, note log scale of *y-axis*). This effect was even more pronounced for SP-FGF-2-GFP as

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0014-5793/$34.00
doi:10.1016/j.febslet.2008.05.042

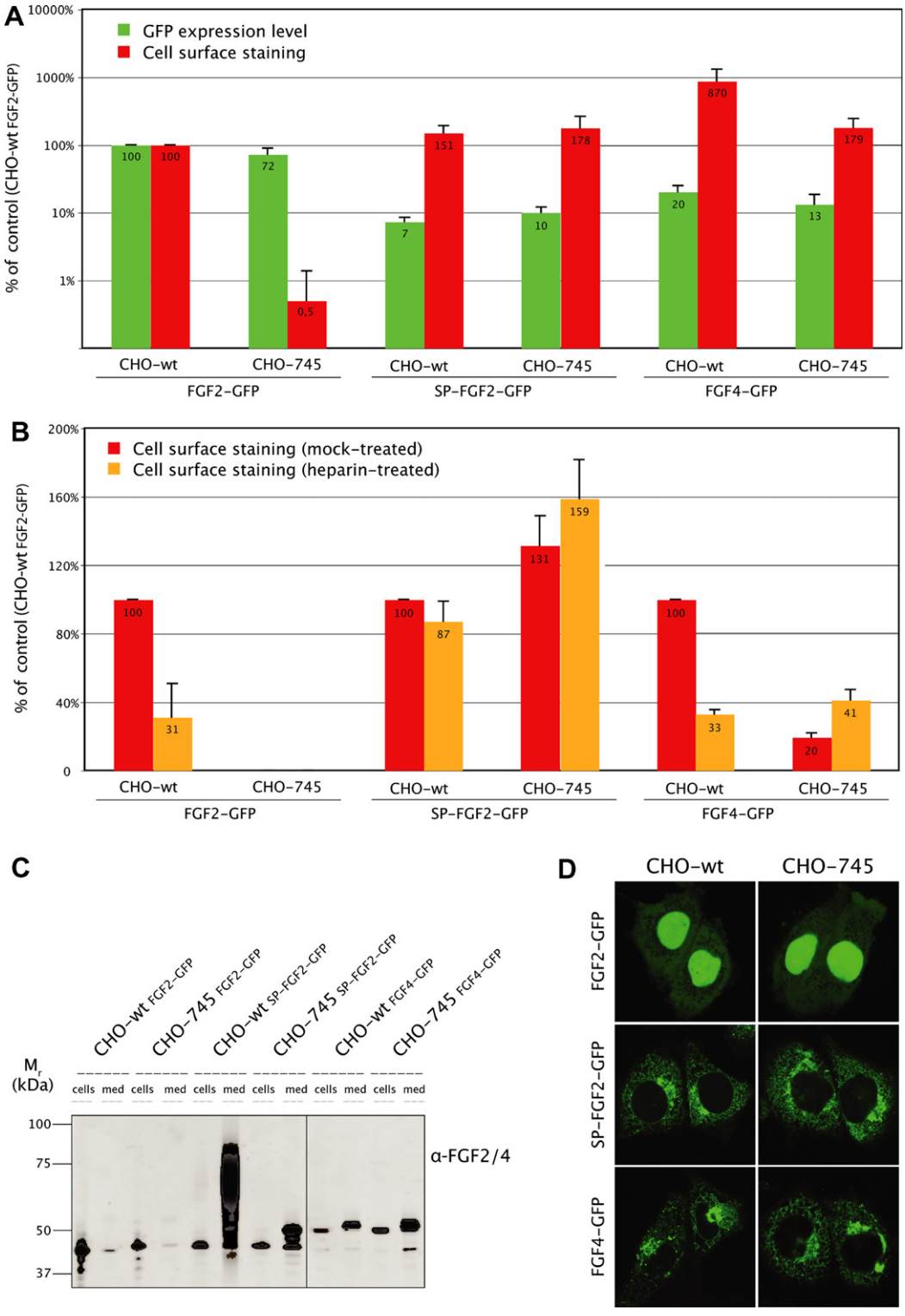

Fig. 1. Rerouting to the ER/Golgi-dependent secretory pathway results in the secretion of aberrant forms of FGF-2. (A) FACS-based quantitation of cell surface-associated material of FGF-2-GFP, SP-FGF-2-GFP and FGF-4-GFP (green bars = GFP expression level; red bars = cell surface staining). All measurements were normalized by setting the levels of FGF-2-GFP to 100%. (B) Heparin-mediated elution of FGF-2-GFP, SP-FGF-2-GFP and FGF-4-GFP from cell surfaces. (C) Biochemical analysis of FGF-2-GFP, SP-FGF-2-GFP and FGF-4-GFP in cell lysates and cellular supernatants. (D) Subcellular localization of FGF-2-GFP, SP-FGF-2-GFP and FGF-4-GFP in CHO wild-type and CHO-745 cells. For further details see text.

similar amounts of this fusion protein were found on the surfaces of CHO wild-type and CHO-745 cells, respectively. Consistently, elution of both SP-FGF-GFP and FGF-4-GFP from the cell surfaces of CHO-745 cells could not be observed (Fig. 1, panel B). These data establish that both SP-FGF-2-GFP and FGF-4-GFP can bind to sites on cell surfaces that are

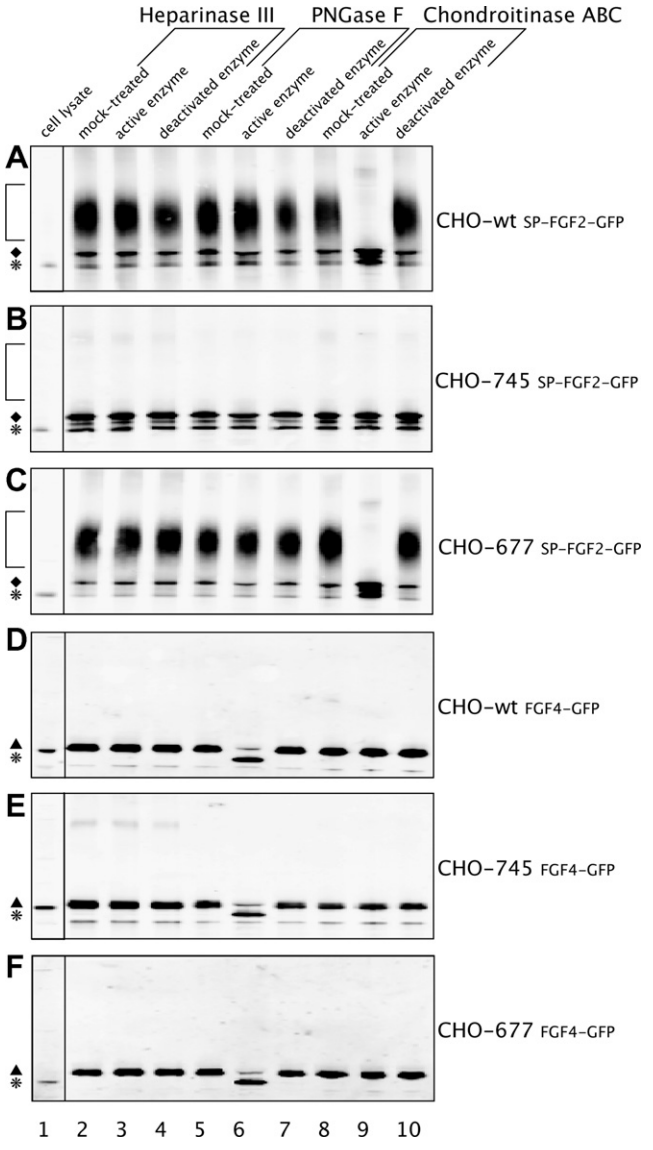

Fig. 2. The high molecular weight forms of SP-FGF-2 ([) originate from the addition of glycosaminoglycan chains of the chondroitin sulfate type. (A) SP-FGF-2-GFP expressed in CHO wild-type cells. (B), SP-FGF-2-GFP expressed in CHO-745 mutant cells. (C) SP-FGF-2-GFP expressed in CHO-677 mutant cells. (D) FGF-4-GFP expressed in CHO wild-type cells. (E) FGF-4-GFP expressed in CHO-745 cells. (F) FGF-4-GFP expressed in CHO-677 cells. Lane 1: cell lysate. Lanes 2–10: Immunoprecipitated material from cellular supernatants. Lanes 2, 5, and 8: Mock-treated samples. Lanes 3, 6, and 9: Samples treated with the enzyme indicated. Lanes 4, 7, and 10: Samples treated with heat-inactivated enzymes. For further details see text.

not provided by HSPGs. The combined data discussed above were taken as a first hint that, as opposed to FGF-2 and FGF-4, SP-FGF-2 could not bind to HSPGs.

As shown by confocal microscopy, addition of a signal peptide to FGF-2 (to generate SP-FGF-2) indeed caused its rerouting to the ER/Golgi system as indicated by the lack of diffuse cytoplasmic and nuclear localization. Instead, like FGF-4, intracellular SP-FGF-2 was localized in perinuclear structures likely to represent the ER/Golgi system (Fig. 1, panel D). As opposed to FGF-2 but similar to FGF-4, SP-FGF-2 was not only found on cell surfaces (Fig. 1, panel A) but rather substantial amounts of SP-FGF-2 were also secreted into the medium of both CHO wild-type and CHO-745 cells (Fig. 1, panel C). As compared to the intracellular population, SP-FGF-2 being secreted into the medium of cells was apparently modified. A broad range of high molecular weight forms were observed that were clearly distinct from unmodified FGF-2 (Fig. 1, panel C). In contrast to SP-FGF-2, the secreted population of FGF-4 found in cellular supernatants was represented by only one major species (Fig. 1, panel C).

2.2. Rerouting of FGF-2 to the classical secretory pathway results in the addition of glycosaminoglycans of the chrondroitin sulfate type

In order to characterize the apparent post-translational modifications of secreted SP-FGF-2, we conducted enzymatic digestions combined with the expression of SP-FGF-2 in CHO wild-type and various mutant cell lines deficient in glycosaminoglycan synthesis (Fig. 2). We compared digestion with heparinase III (to target heparan sulfates; [11]), PNGase F (to target *N*-glycosylation; [12]) and chrondroitinase ABC (to target chondroitin sulfate; [13]) as indicated. When secreted SP-FGF-2 was derived from CHO wild-type cells (Fig. 2, panel A), the high molecular weight forms ([) were neither affected by heparinase III nor by PNGase F, however, treatment with chrondroitinase ABC resulted in its complete digestion (lane 9). Consistently, as shown in panel B of Fig. 2, the high molecular weight forms of SP-FGF-2 were absent from the cell culture supernatants of CHO mutant cells that cannot add any glycosaminoglycan chains to target proteins (CHO-745; [10]). By contrast, CHO mutant cells that cannot transfer heparan sulfates, however, are able to modify target proteins with glycosaminoglycans of the chrondroitin sulfate type (CHO-677; [14,15]) did secrete the high molecular weight forms of FGF-2-GFP which, in turn, were digestible with chrondroitinase ABC (Fig. 2, panel C, lane 9).

In the experiments shown in Fig. 2 (panels A–C), a small fraction of secreted SP-FGF-2 was detected that was still running slower (*υ*; Fig. 2, lanes 2–10) than the material found in cell lysates (*ι*; Fig. 2, lane 1). This modified form of SP-FGF-2 was neither affected by heparinase III, PNGaseF nor by chrondroitinase ABC treatment. It was secreted from both CHO wild-type as well as from CHO-745 and 677 mutant cells. These findings suggest that, in addition to receiving glycosaminoglycans of the chrondroitin sulfate type, SP-FGF-2 becomes *O*-glycosylated at another site resulting in the low molecular weight form (*υ*).

As opposed to SP-FGF-2, secreted FGF-4 was represented by just one discrete form (*σ*; Fig. 2, panels D–F, lanes 2–5 and 7–10) that was running more slowly compared to the material found in cell lysates (*ι*; Fig. 2, panels D–F, lane 1). As shown in Fig. 2, the secreted form of FGF-4 was unaffected in the presence of both heparinase III and chrondroitinase ABC. By contrast, this population could be digested with PNGaseF suggesting that secreted FGF-4 derived from CHO cells is *N*-glycosylated which is in line with previous reports [16]. Consistently, the modified form of FGF-4 was secreted from all CHO cell lines being tested.

2.3. SP-FGF-2 fails to bind both heparin and heparan sulfate proteoglycans

In a third set of experiments we tested whether secreted SP-FGF-2 binds to cell surface HSPGs and heparin. To systematically compare FGF-2, SP-FGF-2 and FGF-4 we developed a

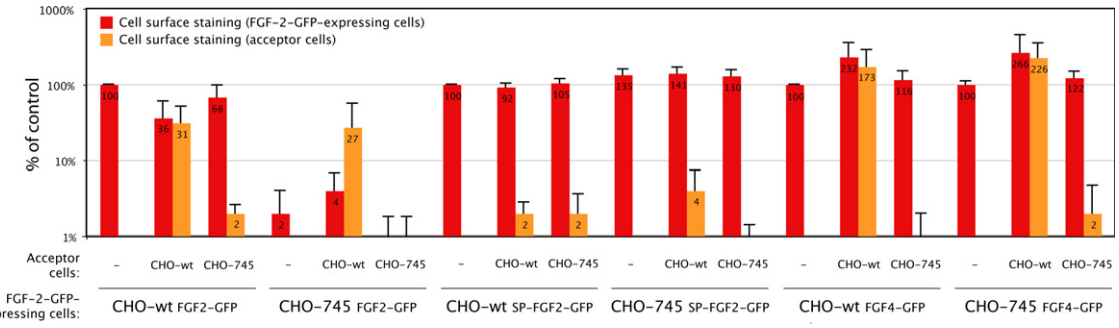

Fig. 3. SP-FGF-2 is deficient in binding to cell surface HSPGs. FGF-2-GFP, SP-FGF-2-GFP, and FGF-4-GFP, respectively, were expressed in CHO wild-type and CHO-745 cells and co-cultivated with the various CHO acceptor cells as indicated. Cell-surface-associated material from FGF-2-GFP-expressing CHO wild-type cells cultivated as a homogenous cell population was used to normalize all data. Red bars indicate cell-surface-associated material of cells expressing the reporter molecules noted above. Orange bars indicate the amounts of cell-surface-associated material on CHO acceptor cells. For further details see text.

co-cultivation setup to measure HSPG-dependent binding to cell surfaces (Fig. 3). This was achieved by co-cultivation of CHO acceptor cells that do not express any of the FGFs being studied. As a control we also used HSPG-deficient CHO-745 cells as acceptors. Both the material on the surface of the FGF-expressing cells (red bars) and the material on the surface of the CHO acceptor cells (orange bars) were quantified by flow cytometry. The signals were normalized by quantification of the cell surface signal of FGF-2-expressing CHO wild-type cells grown as a homogenous culture. When FGF-2 expressing CHO wild-type cells were mixed with CHO wild-type acceptor cells efficient transfer was observed. In case CHO-745 cells were used, transfer of FGF-2 was almost completely abolished demonstrating that transfer to CHO wild-type cells was dependent on HSPGs. As reported previously [7], the FGF-2 secretion deficiency of CHO-745 cells could be overcome by co-cultivation with CHO wild-type cells exposing HSPGs on their cell surfaces. Strikingly, as shown in Fig. 3, expression of SP-FGF-2 in both CHO wild-type and CHO-745 cells did not result in transfer to CHO acceptor cells irrespective of whether the latter were CHO wild-type or CHO 745 mutant cells. By contrast, secretion of FGF-4 from both CHO wild-type and CHO-745 cells did result in efficient transfer to CHO wild-type cells, however, there was no signal when CHO-745 cells were used as acceptors. These data demonstrate that FGF-2 secretion via the classical ER/Golgi-dependent pathway results in the failure of the secreted molecule to bind to cell surface HSPGs. By contrast, both non-classical secretion of FGF-2 and secretion of FGF-4 via the classical secretory pathway results in secreted molecules that are functional with regard to binding to cell surface HSPGs.

These data were confirmed by experiments investigating the binding of SP-FGF-2 and FGF-4 to heparin in vitro (Fig. 4). Similar to recombinant FGF-2-GFP and a non-tagged form of FGF-2 that were used as positive controls (Fig. 4, panels E and F, respectively, lane 2), secreted FGF-4 derived from both CHO wild-type and CHO-745 mutants cells could be shown to efficiently bind to heparin (Fig. 4, panels C and D, lane 2). By contrast, irrespective of whether secreted from CHO wild-type or from CHO 745 mutant cells (Fig. 4, panels A and B, lane 2), binding of SP-FGF-2-GFP to heparin could not be observed. These experiments also demonstrated that the GFP tag in all fusion proteins used throughout this study does not affect binding efficiency to heparin. From the combined data shown in Figs. 3 and 4 we conclude that rerouting of FGF-2 to the ER/Golgi-dependent secretory pathway results in secreted molecules that cannot bind to heparan sulfate proteoglycans.

3. Discussion

We have addressed the question as to why FGF-2 takes an alternative secretory route that does not rely on the classical ER/Golgi-dependent pathway. Consistent with earlier studies [17,18], we demonstrate that FGF-2 is efficiently secreted when artificially targeted to the lumen of the ER (SP-FGF-2). These findings suggest that a general incompatibility of FGF-2 with transport along the classical secretory pathway does not exist. We found, however, that the vast majority of the secreted population of SP-FGF-2 is post-translationally modified by O-linked glycosaminoglycans and that these forms of SP-FGF-2 fail to bind to cell surface HSPGs, an interaction that is critically important for both FGF-2 storage in the extracellular space and FGF-2-mediated signal transduction.

Based on the expression in various CHO mutant cell lines and combined with enzymatic digestions we demonstrate that the secreted population of SP-FGF-2 is *O*-glycosylated. In particular, in cellular supernatants, high molecular weight forms of SP-FGF-2 accumulate that are generated by the addition of glycosaminoglycan chains. These aberrant forms of FGF-2 are not secreted from CHO-745 mutant cells [10], a cell line with a general block in glycosminoglycan synthesis. By contrast, in case of CHO-677 cells [14,15], a cell line that cannot produce heparan sulfates, however, is capable of synthesizing glycosaminoglycans of the chrondroitin sulfate type, the high molecular weight forms of SP-FGF-2 are present in cellular supernatants. Consistently, enzymatic digestion with chondroitinase ABC resulted in the degradation of these modified forms of SP-FGF-2. The combined data establish that, when FGF-2 is targeted to the ER/Golgi system via an artificial signal peptide, it becomes modified with glycosaminoglycan chains of the chondroitin sulfate type and is secreted efficiently.

In the second part of this work we found that, as opposed to the normal 18 kDa form of FGF-2, SP-FGF-2 modified with glycosaminoglycans looses its ability to bind to both HSPGs and heparin. This was shown by co-cultivation experiments using living cells and by in vitro studies analyzing binding

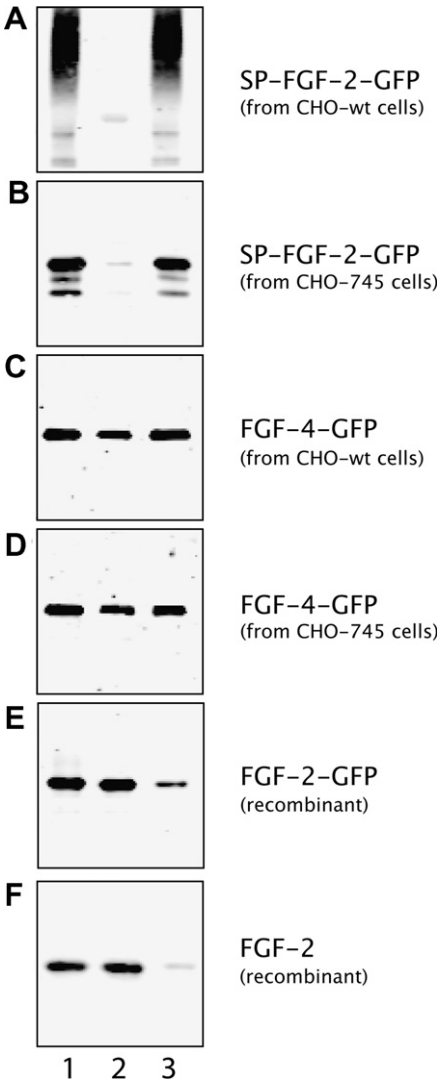

Fig. 4. SP-FGF-2 is deficient in binding to heparin in vitro. Cell culture supernatants either derived from CHO wild-type (panels A and C) or CHO-745 mutant cells (panels B and D) expressing SP-FGF-2-GFP (panels A and B) and FGF-4-GFP (panels C and D), respectively, were incubated with immobilized heparin. As positive controls, recombinant FGF-2-GFP and a non-tagged form of recombinant FGF-2 were used (panels E and F, respectively). Lane 1: Input (50% following immnuoprecipitation). Lane 2: Bound fraction (50%). Lane 3: Flow-through fraction (50% following immnuoprecipitation).

capabilities to immobilized heparin. While we demonstrated efficient binding of FGF-2 secreted by the normal alternative pathway, modified SP-FGF-2 completely failed to bind to both cell surface HSPGs and heparin. In all of these studies, we systematically compared FGF-2 and SP-FGF-2 with FGF-4, a close relative of FGF-2 that naturally contains a signal peptide and is secreted via the ER/Golgi-dependent pathway. Interestingly, even though structurally related [19], FGF-4 does not receive O-linked glycosaminoglycans within the lumen of the ER/Golgi system. Rather, we show that the secreted population of FGF-4 is *N*-glycosylated, a modification that is compatible with binding to both cell surface HSPGs and heparin.

A major role of the interaction between FGF-2 and HSPGs on cell surfaces and in the extracellular matrix is storage and protection against degradation and denaturation of extracellular FGF-2 [20–22]. In the light of the current data, it becomes evident that this physiological function of HSPGs is not compatible with the secretion of FGF-2 based on the ER/Golgi-dependent pathway. Additionally, FGF-2-mediated signal transduction in most cases depends on ternary complexes of FGF-2, HSPGs and high affinity FGF receptors [23]. Our data are therefore consistent with previous studies demonstrating that FGF-2 secreted from the ER/Golgi-dependent pathway has no or only little mitogenic activity [17]. Therefore, as opposed to what has been concluded in a recent study [24], it remains questionable whether signal-peptide-containing forms of FGF-2 could be relevant as a therapeutic tool to deliver large amounts of FGF-2 in a functional form. In conclusion, our data suggest that the unconventional secretory pathway of FGF-2 represents an ancient mechanism of protein secretion that, in the course of evolution, was not replaced by the more complex, ER/Golgi-dependent pathway to export FGF-2. Our findings suggest that this is due to the inability of the ER/Golgi-dependent pathway to secrete FGF-2 in a physiologically active form.

4. Materials and methods

4.1. Reagents and cell lines

A signal-peptide-containing form of FGF-2 (SP-FGF-2) was generated by transplanting the natural FGF-4 signal peptide (MSGPGTAAVALLPAVLLALLAPWAGRGGAAAPTAP) to the N-terminus of the 18 kDa form of FGF-2. Stable CHO wild-type, CHO-745 [10] and CHO-677 [14,15] mutant cell lines, respectively, were generated by retroviral transduction as described before [25]. Antibodies used for flow cytometry and biochemical experiments were directed against GFP (monoclonal, Clontech), FGF-2 (monoclonal, clone FB-8, Sigma), and FGF-4 (monoclonal, clone 19805.11, Abcam).

4.2. Quantification of FGF-2 secretion based on flow cytometry

Stable cell lines expressing FGF-2, SP-FGF-2, and FGF-4, respectively, in a doxicycline-dependent manner were used to quantify overall expression levels (GFP fluorescence) and cell-surface-exposed material by antibody staining as described before [25,7]. Where indicated cells were treated with heparin (125 μg/ml) to quantify cell surface-associated material that was bound to HSPGs.

4.3. Biochemical analysis of FGF-2 secretion

Cells were grown in six-well plates to about 80% confluency. To analyze the appearance of FGF-2, SP-FGF-2, and FGF-4, respectively, we immunoprecipitated them from cellular supernatants followed by SDS–PAGE (Invitrogen NuPage, 4–12% Bis–Tris gel using MOPS SDS running buffer) and Western blot analysis using monoclonal antibodies directed against FGF-2 and FGF-4, respectively. Fifty percent of the immunoprecipitates were compared to about 1% of the corresponding cell lysates.

4.4. Confocal microscopy

CHO cells were grown on glass coverslips (MatTek Corp. Ashland, USA), processed for live cell imaging and viewed with a Zeiss LSM 510 confocal microscope.

4.5. Analysis of post-translational modifications by enzymatic digestions

Cells were grown in six-well plates to about 80% confluency. SP-FGF-2 and FGF-4, respectively, were immunoprecipitated from the cellular supernatants of the cell lines indicated and 50% of each immunoprecipitate were analyzed by SDS–PAGE (Invitrogen NuPage, 4-12% Bis–Tris gel using MOPS SDS running buffer) and Western blot analysis. Where indicated, immunoprecipitates were treated with heparinase III (from `Flavobacterium heparinum`; `Sigma H8891`), PNGase F (`Peptide-N-glycosidase F`; Sigma P7367) and

chondroitinase ABC (Chondroitinase ABC from Proteus vulgaris; Sigma C3667). As controls, immunoprecipitates were mock-treated or incubated with heat-inactivated enzymes. In each case, cell lysates (0.5%) were analyzed for comparison.

4.6. Quantification of FGF-2 binding to cell surface HSPGs by co-cultivation experiments

Binding of secreted FGF-2, SP-FGF-2, and FGF-4-GFP, respectively, to cell surface HSPGs was measured by co-cultivation experiments using various kinds of CHO acceptor cells as described previously [7].

4.7. Analysis of FGF-2 binding to heparin in vitro

Cells were grown in six-well plates to about 80% confluency. The cellular supernatants of CHO wild-type and CHO-745 cells expressing SP-FGF-2-GFP and FGF-4-GFP, respectively, were incubated with heparin-coated beads (Heparin Sepharose 6 Fast Flow, GE Healthcare). Bound and unbound material was separated and 50% of each fraction were analyzed by SDS–PAGE (12%) and Western blot analysis. Input material was generated by direct immunoprecipitation of SP-FGF-2-GFP and FGF-4-GFP from the corresponding cellular supernatants. As a positive control, recombinant FGF-2-GFP (0.6 μg) was used.

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